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Tracking Origins of Prostate Cancer: An Innovative *in Xivo* Modeling

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14. ABSTRACT Heterogeneity, variable and often unpredictable clinical course are fundamental challenges in management of patients with prostate cancer. To make rapid advances in understanding of disease mechanism that can be translated to clinical care in short order, there is an immediate need for innovative in vivo disease models that accurately recapitulate human disease at cellular level. We propose to develop an innovative and hitherto not attempted in vivo prostate cancer model that will delineate the exact cell of origin through different stages of prostate cancer development and progression. We propose to study possible cell(s) of origin for prostate cancer by combinatorial expression of florescent magenta, cyan and yellow primary color proteins in prostate at development in mice. The study includes (1) Construction of "Prorainbow" plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters. (2) Establish mouse line with the resulting "Prorainbow" construct and generation of transgenic mice by crossing with Cre mice. (3) Study the transgenic Prorainbow mice under normal and oncogenic conditions. The study is expected to produce in vivo animal of prostate cancer with unique capabilities.					
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Final Report

Title: Tracking Origins of Prostate Cancer – an Innovative *in vivo* Modeling

Table of Contents

	<u>Page</u>
Cover.....	1
SF 298 Form.....	2
Introduction.....	4
Accomplishments.....	4
References.....	26
Impact.....	26
Changes/Problems.....	26
Products.....	26
Participants and other collaborating organizations.....	27
Special reporting requirements.....	27
Appendix.....	28

Introduction

Prostate cancer is the most frequently diagnosed cancer of men and the most common cancer overall (1). The cancer cells may metastasize from the prostate to other parts of the body, particularly the bones and lymph nodes. About 20% of patients undergoing radical prostatectomy develop metastasis beyond 5 years, suggesting metastasis is an early event and removal of primary tumor does not significantly decrease the rate of metastasis. Thus, understanding the role of the genetic changes leading to origination and development of primary tumor and metastasis would provide for a targeting strategy to clinical therapy. The goal of this project is to study the origin of cancer cells within the prostate. Since development of human prostate cancer proceeds through a series of defined states, we would utilize a newly developed fluorescent protein labeling technique, Brainbow, which has been used to study the nervous system development in Brain (2). Similar to the 'Brainbow' concept we propose 'Prorainbow' modeling to track prostate cell proliferation and differentiation by labeling individual early prostate precursor cell a unique color. In case of a tumor or metastasis, we can track down the ancestor normal cell by matching to the tumor cell color. We can then track these color distributions and pattern changes with time course, which will build up a dynamic vision of prostate cancer progression. Also, we want to examine functions of Protein Kinase D1 (PKD1) and Phosphatase and Tensin homolog (PTEN) in conditional knockout mice in the development of cancer formation and metastasis in the prostate. Successful development of fluorescent labeled *in vivo* animal model will be unique in the field of prostate cancer research and provide much needed advance to understand progression of prostate cancer.

We proposed to testify the stated hypothesis with following aims:

- 1) Construction of 'Prorainbow' plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters.
- 2) Establish mouse line with the resulting 'Prorainbow' construct and generation of transgenic mice by crossing with Cre mice.
- 3) Study the transgenic Prorainbow mice under normal and oncogenic conditions.

Keywords

Prostate cancer, *in vivo* model, Prorainbow, tumor development

Accomplishments

Aim (1) Construction of 'Prorainbow' plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters.

Task I. Generation of Probasin promoter controlled XFP.

Probasin is a prostate specific and androgen-regulated protein, which can be used as a marker of prostate differentiation. The rat probasin promoter (ARR2PB), which is 455 bp in size, has been successfully cloned and used in transgenic mice to target high-level, prostate-specific expression of down-stream transgenes², and the expression regulated by Cre recombinase is in both basal and luminal epithelial cells. We amplified the PB promoter by PCR from pPr-luc (Addgene #8392) and specific primers (PB-F: 5'-AGTCATT AATAAGCTTCCACAAGTGCATTTAGCCTCTCC-3'; PB-R: 5'- AGTCGCTAGCCTGTAGGTATCTGGACCT CACTGAC-3') (Figure1), and successfully ligated the promoter into Brainbow 1.0L vector to replace the original CMV promoter (Figure1). We checked the DNA plasmids by restriction enzyme digestion (Fig 2A) and DNA sequencing by primers (5' Asel check: 5'-AGCCTATGGAAAAACGCCAG-3'; 3' NheI check: 5'-ATCAAAGAGTTCATGCGCTT-3') to make sure the promoter sequence is intact and of no point mutations.

We confirmed that cells transfected with those plasmids can express fluorescent proteins under the control of Probasin promoter by testing the expression of this construct in human prostate LnCap cells (Fig 3A), as well as the expression in other human cell lines (NIH 3T3, HEK 293, MCF7, MS1, normal kidney primary cells and smooth muscle primary cells).

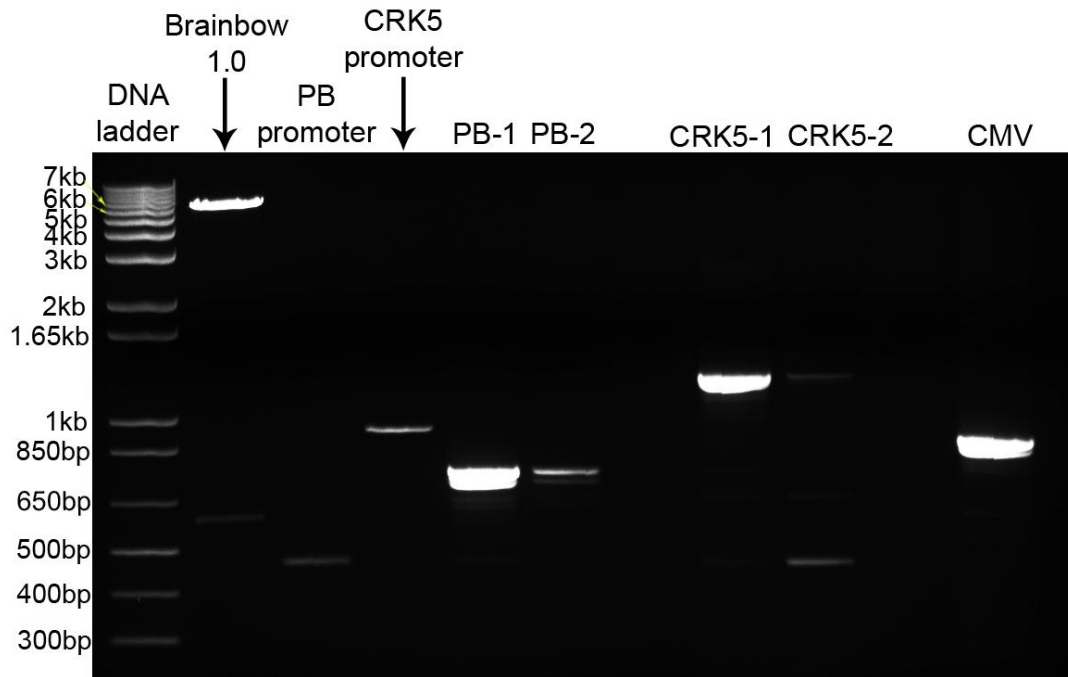


Figure 1 Subcloned Probasin promoter and Cytokeratin 5 promoter into the Brainbow vector. Brainbow 1.0, the Brainbow 1.0L plasmid digested by *Asel* and *NheI*. The upper band is the backbone (6.28kb), and the lower band is the CMV promoter (574bp). PB promoter, the probasin promoter amplified by PCR using primers PB-F and PB-R (expected size 475bp). CRK5 promoter, the Cytokeratin 5 promoter amplified by PCR using primers CRK5-F and CRK5-R (expected size 927bp). PB-1, PB-2, the probasin promoter and extension area amplified by PCR (templates are PB-Brainbow candidate clone 1 and 2, and the primers are 5' *Asel* check and 3' *NheI* check) (expected size 740bp). CRK5-1, CRK5-2, the cytokeratin 5 promoter and extension area amplified by PCR (templates are CRK5-Brainbow candidate clone 1 and 2, and the primers are 5' *Asel* check and 3' *NheI* check) (expected size 1212bp). CMV (control), the CMV promoter and extension area amplified by PCR (template is Brainbow 1.0L, and the primers are 5' *Asel* check and 3' *NheI* check) (expected size 865bp).

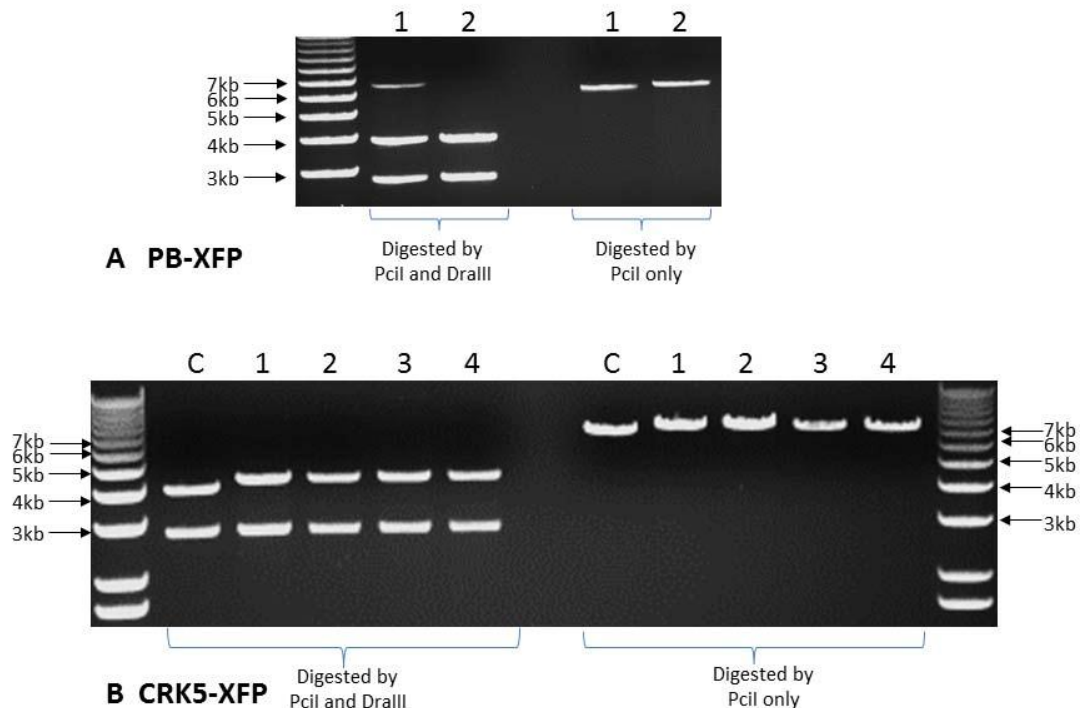


Figure 2 PB-XFP and CRK5-XFP DNA constructs were successfully produced. In A, 1 and 2 are DNA construct candidates for PB-XFP. 2 is of correct size, checked by single digestion with *PciI* (right two lanes) or double digestion with both *PciI* and *DraIII* (left two lanes). In B, 1~4 are DNA construct candidates for CRK5-XFP, and all four of them are of correct size, checked by single digestion with *PciI* (right panel) or double digestion with both *PciI* and *DraIII* (left panel). C

is the control DNA construct which is CMV-XFP (Brainbow 1.0L). Expected sizes: linearized PB-XFP (single digested by PciI), 6737bp; linearized CRK5-XFP (single digested by PciI), 7179bp; linearized CMV-XFP (single digested by PciI), 6856bp; transgene with Probasin promoter (digested by PciI and DrallI), 3686bp; transgene with Cytokeratin 5 promoter (digested by PciI and DrallI), 4128bp; backbone of the plasmid (digested by PciI and DrallI), 3051bp.

Task II. Generation of Cytokeratin 5 promoter controlled XFP.

Cytokeratin (CRK) 5 is expressed specifically in basal layer of all stratified squamous epithelia³⁻⁵. After the differentiation, CRK5 expression would be gradually lost⁶, which makes CRK5 promoter an ideal candidate for direction of XFP expression. We amplified the CRK5 promoter (907 bp) by PCR using human genomic DNA as template and specific primers (CRK5-F: 5'- AGTCATTAATGATCCCCGGGTTTCCTAAACC-3'; CRK5-R: 5'- AGTCTCTAGA GGCTTGTTCTGGTGGAGCAAGAGAAC-3') (Figure1), and successfully ligated the promoter into Brainbow 1.0L vector to replace the original CMV promoter (Figure1). We checked the DNA plasmids by restriction enzyme digestion (Fig 2B) and DNA sequencing using primers (5' AseI check: 5'-AGCCTA TGGAAAACGCCAG-3'; 3' NheI check: 5'-ATCAAAGAGTTCATGCGCTT-3') to make sure the promoter sequence is intact and of no point mutations.

We confirmed that cells transfected with those plasmids could express fluorescent proteins under the control of Cytokeratin 5 promoter by testing the expression of this construct in human prostate LnCap cells (Fig 3B), as well as the expression in other human cell lines (NIH 3T3, HEK 293, MCF7, MS1, normal kidney primary cells and smooth muscle primary cells).

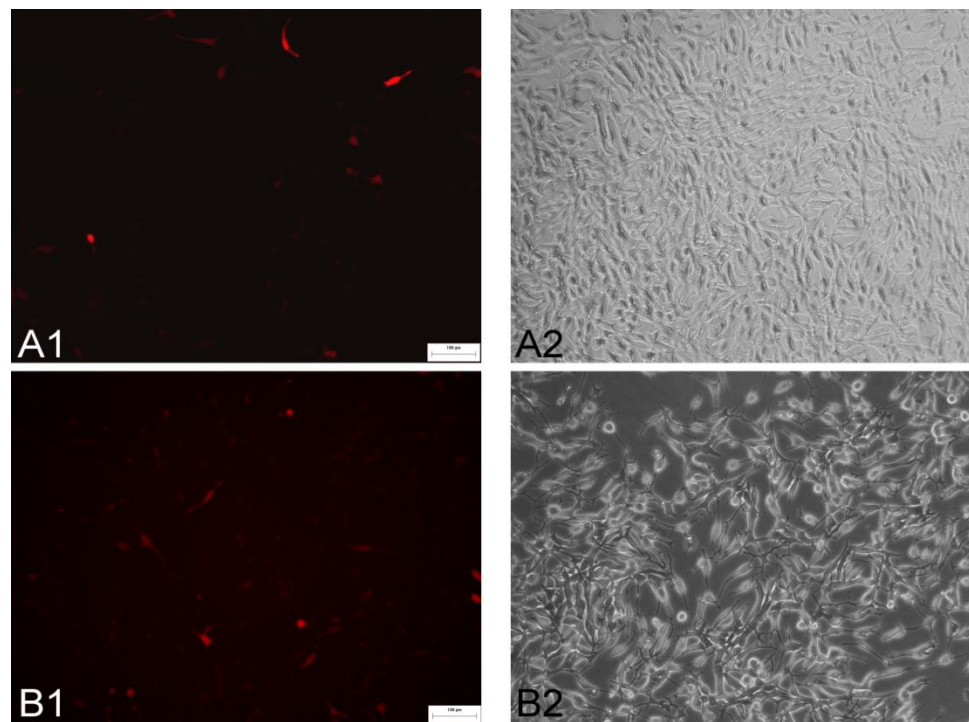


Figure 3 Transfection of Prorainbow constructs in LnCap cells. A, PB-XFP transfection. A1 is red fluorescent view and A2 is bright field view. B, CRK5-XFP transfection. B1 is red fluorescent view and B2 is bright field view. Scale bar, 100 μ m.

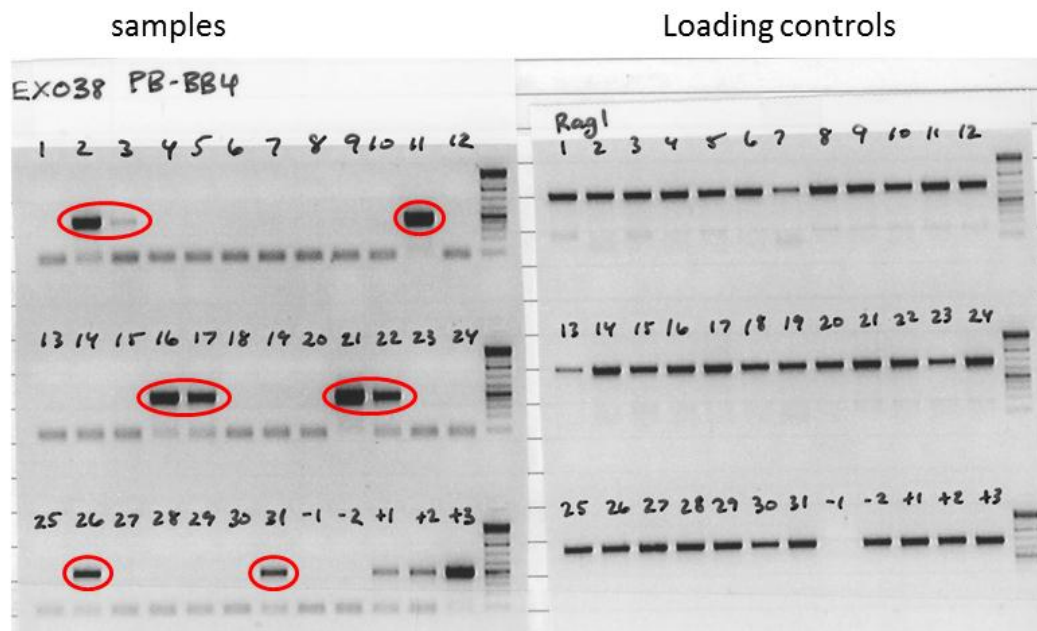
Aim (2) Establish mouse line with the resulting ‘Prorainbow’ construct and generation of transgenic mice by crossing with Cre mice.

Task I. Obtain institutional approval for animal study.

Since the PI and the whole lab moved to Wake Forest University (WFU) in 2010, we submitted a new animal protocol at WFU to get the permission of carrying out all the experiments proposed. The animal protocol is approved by Institutional Animal Care and Use Committee (IACUC) at WFU (Protocol # A11-097). Later we submitted an animal use appendix to USAMRMC Animal Care and Use Review Office (ACURO) and got the granted approval from ACURO (Appendix1).

Task II. Generation of Prorainbow construct expressing transgenic mice (n=3~5).

We prepared digested DNA fragments of Prorainbow constructs and the pronuclear injection was done by University of North Carolina (UNC) Animal Models Core. Nine PB-XFP founder animals (Fig.4) and eight CRK5-XFP founder animals were generated after genotyping (Fig. 5). Primers RPBPF (5'-TCTGATTGGAGGAATGGATAATAGTCATC-3') and tdTomato-R2 (5'-CACCTTGAAGCGCATGAACTCTTTGATG-3') were used for PB-XFP founder sequencing, and primers HCK5P-F (5'-GCAAGGCAAGGTTATTTCTAACTGAGCA-3') and tdTomato-R2 (5'-CACCTTGAAGCGCATGAACTCTTTGATG-3') were used for CRK5-XFP founder sequencing. Mouse *Rag1* primers F2 (5'-TTCTGCCGCATCTGTGGGAATC-3') and R2 (5'-CTTCACATCTCCACCTTCTTCTTTGTCAG-3') were used in PCR as DNA loading controls. The PCR cycles used in genotyping were: 95 °C, 2 min; 95°C, 30 sec, 58 °C, 30 sec, 72 °C, 1min, 35 cycles; 72°C, 10min.



Conclusion: animals #2, 3, 11, 16, 17, 21, 22, 26 and 31 are positive for the transgene

Figure 4 Genotyping results of PB-XFP founder mouse candidates. Samples:#1-21, samples from potential founder animals. Controls: -1, dH₂O template; -2, wt DNA; +1, PB-XFP transgene diluted in wt DNA at 0.1 copy/genome; +2, PB-XFP transgene 1 copy/genome; +3, PB-XFP transgene 10 copies/genome. Left panel used primer pair RPBPF and tdTomato-R2 in PCR, and expected product size is 492bp; right panel used primer pair Mouse *Rag1* primers F2 and R2 in PCR, and expected product size is 960bp.

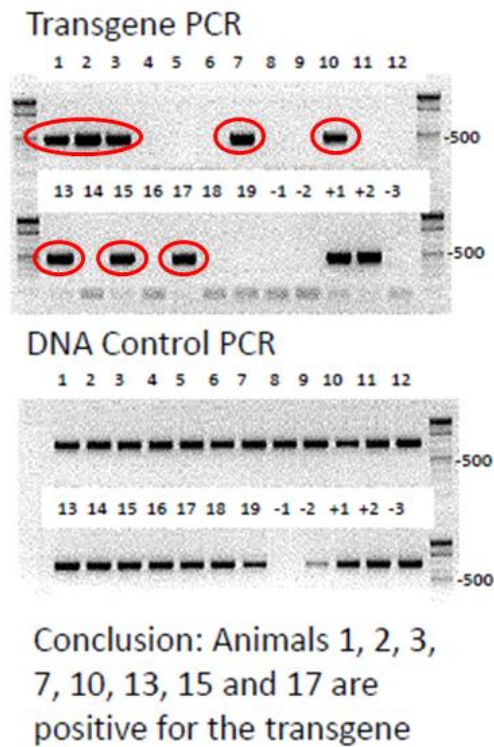


Figure 5 Genotyping results of CRK5-XFP founder mouse candidates. Samples:#1-19, samples from potential founder animals. Controls: -1, dH₂O template; -2, wt DNA; +1, CRK5-XFP transgene 1 copy/genome; +2, CRK5-XFP transgene 10 copies/genome; -3, PB-XFP transgenic animal DNA. Left panel used primer pair hCK5P-F and tdTomato-R2 in PCR, and expected product size is 452bp; right panel used primer pair Mouse *Rag1* primers F2 and R2 in PCR, and expected product size is 960bp.

Task III. Cross-breeding of Cre mice with Prorainbow transgenic mice.

Three different crossings are planned to be set up to validate the Prorainbow system and to study normal prostate development.

- 1) ROSA-CreER X PB-XFP
- 2) PB-Cre4 x CRK5-XFP
- 3) PB-Cre4 x CMV-XFP

After the founder mice (nine PB-XFP animals and eight CRK5-XFP animals) were transferred to Wake Forest University and passed the quarantine requirements, they were bred with Cre-mice (ROSA-Cre or PB-Cre) to test the expression level of fluorescent proteins in prostate.

For the first breeding method, ROSA-CreER mice were ordered from JaxMice (stock 008463, B6.129-Gt(ROSA)26 Sor tm1(cre/ERT2)Tyj/J) and arrived at our laboratory. For PB-XFP founders, six out of nine lines (#511, 512, 513, 517, 518 and 520) were kept for testing. Line #514 was lost due to low transgene inheritance (the female founder gave birth to three litters and all the pups screened were wild type). Line 515 was lost due to health concerns (the female founder had severe malocclusion, and was found dead a few weeks after arrival). Line 516 was lost due to fighting (the male founder was severely wounded by the breeding female mouse and the lesion didn't recover well, which resulted in its euthanasia). The remaining lines were bred with ROSA-Cre animals using specific schemes (Fig.6A, 6D), and we got male pups with correct genotypes for five lines (Fig.7A, mouse Line #511 as an example).

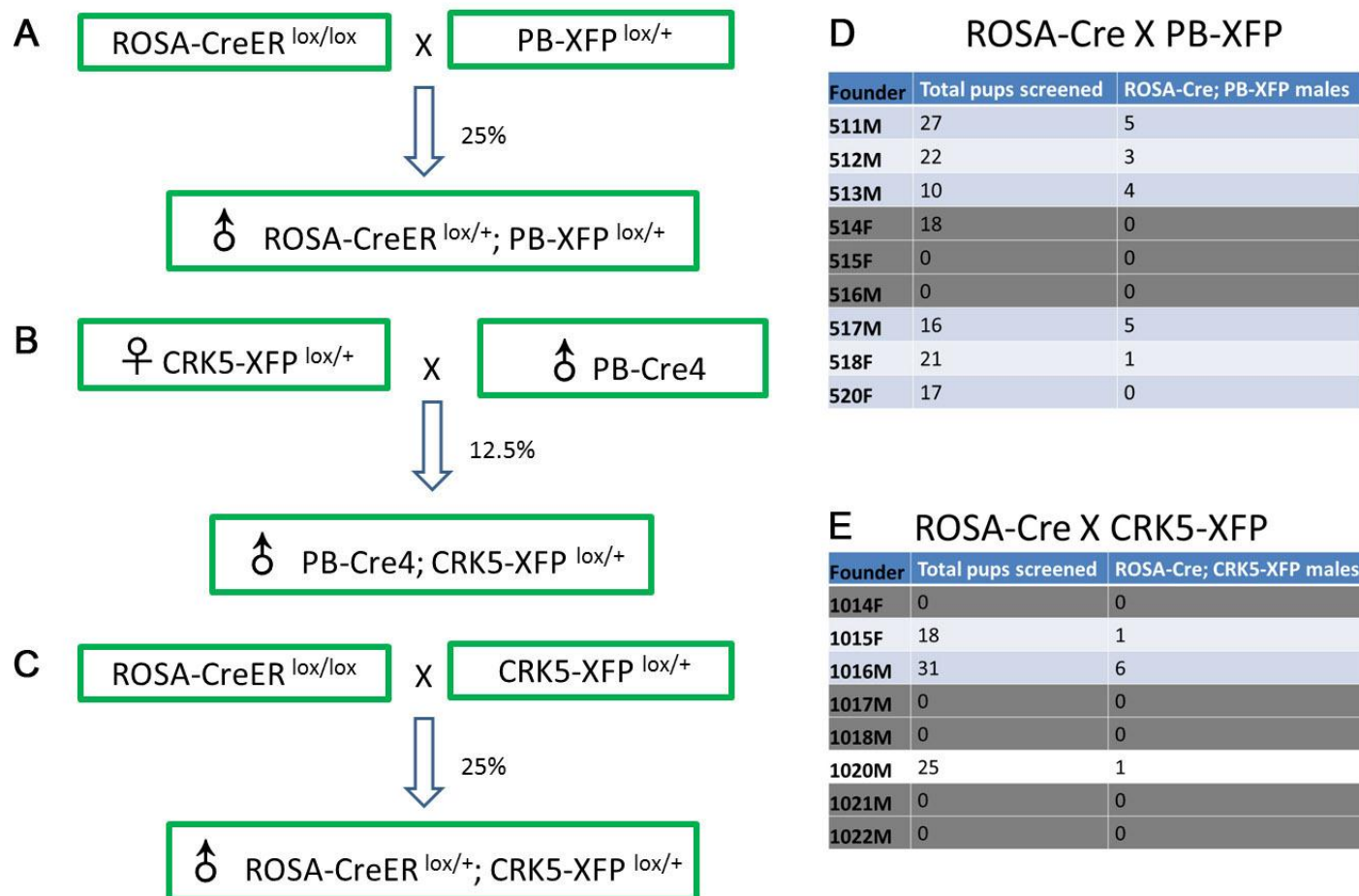


Figure 6 Breeding schemes for transgenic animals carrying PB-XFP and CRK5-XFP. A, breeding between ROSA-CreER and PB-XFP animals. B, breeding between PB-Cre4 males and CRK5-XFP females. C, breeding between ROSA-CreER and CRK5 animals. D, summary of screening results of ROSA-CreER; PB-XFP animals. E, summary of screening results of ROSA-CreER; CRK5-XFP animals.

loxP insertion (PKD1#2, 5'-TGTTCTCCCCAGTGGCAT-3'; PKD1 #3 rev, 5'-AAACGGAAATGCTCACAGAAATAT-3'). We generated PKD1 PTEN double knockout (specifically knocked out in prostate) mice by crossing PB-Cre4; PKD1^{lox/lox} and PTEN^{lox/lox} mice. We got animals of expected genotype (Fig.10). Primers oIMR 9554 and oIMR 9555 were used for genotyping of PTEN loxP insertion (oIMR9554, 5'-CAAGCACTCTGCGAACTGAG-3'; oIMR9555, 5'-AAGTTTTTGAAGGCAAGATGC-3').

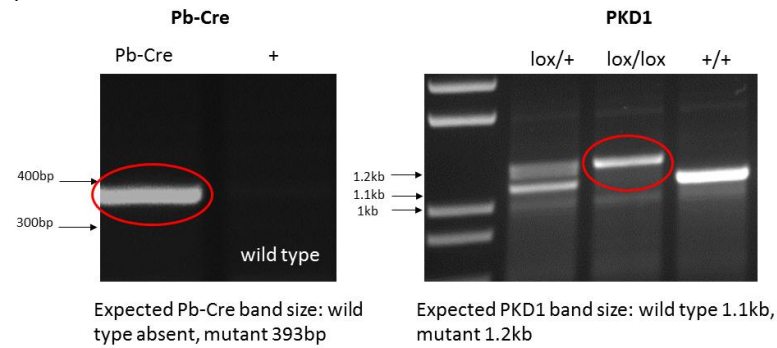


Figure 9 Genotyping results of PKD1 knock-out mouse. Red circles indicating the expected genotype of PKD1 knock-out animal, which is Pb-Cre; PKD1 lox/lox.

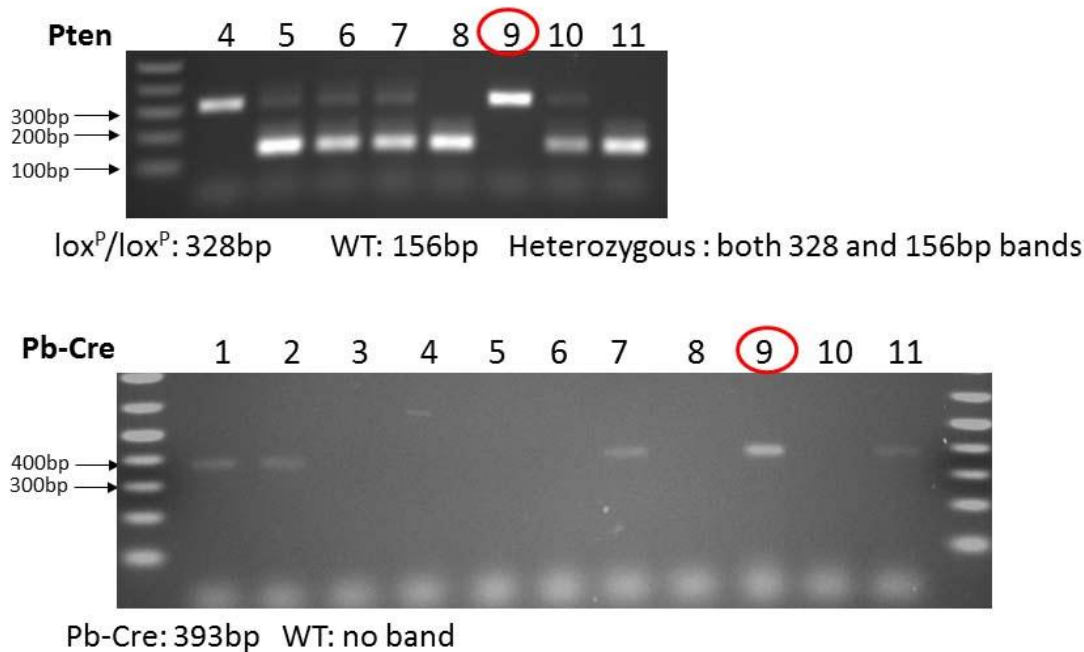


Figure 10 Genotyping results of PKD1 PTEN double knock-out mouse. Mouse #9 (highlighted by red circle) has the expected genotype of double knock-out animal.

After several rounds of breeding, we got knock-out animals (both PKD1 knock-out and PKD1-PTEN double knock-out) with CMV-XFP (Brainbow 2.1) (Fig. 11).

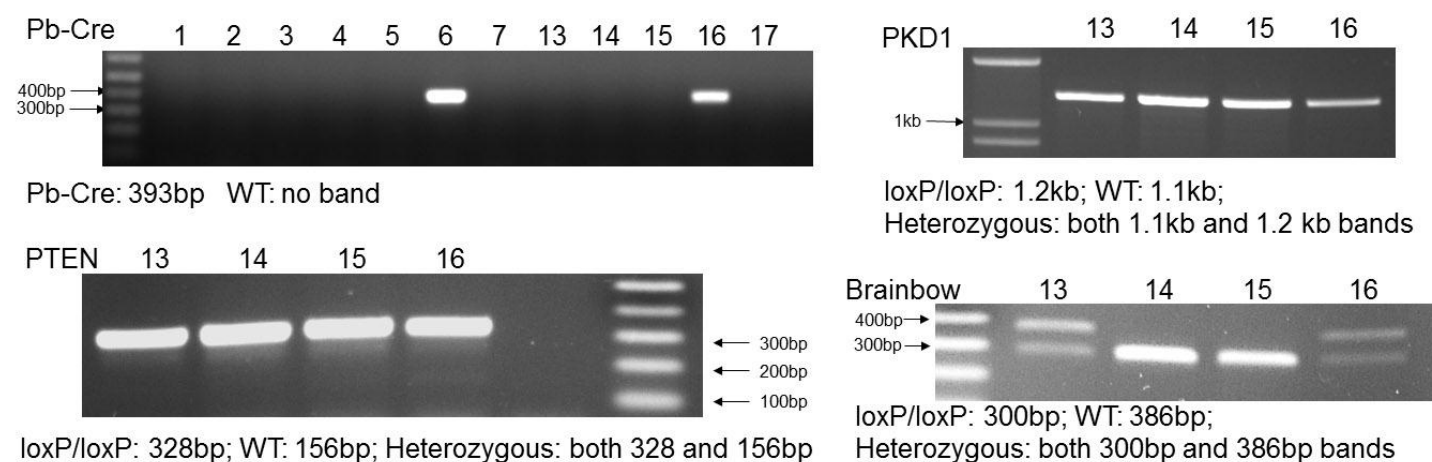


Figure 11 Screening for PKD1 PTEN double knock-out animal with CMV-XFP (Brainbow 2.1). Mouse#16 is a positive candidate.

Aim (3) Study the transgenic Prorainbow mice under normal and oncogenic conditions.

Task 1. Evaluate combinatorial expression of XFP in prostate of Prorainbow mice.

For ROSA-Cre X PB-XFP breeding, we got ROSA-Cre; PB-XFP males from all of the six remaining founder lines. We induced ROSA-Cre expression in adult males with Tamoxifen injection (10mg/ml in corn oil, 100µl/adult/day, for five consecutive days). We checked prostate for XFP expression in all of them, and none of them showed epithelial expression of fluorescent proteins (Fig. 12). We did observe some fluorescent signals in stromal cells. To check whether those signals are background noise, we harvested prostate from non-induced control animal (littermate with same genotype) and did similar check (Fig.13). We also checked signals in kidney of induced animal, as well as that of non-induced control (Fig.13). No fluorescent signal was detected in epithelial cells in either prostate or other organs (Fig.12, 13). Weak background signals were observed from neighboring stromal cells (non-epithelial cells) in the prostate tissue and from kidney cells in induced animals (Fig. 12, 13). The screening results were summarized in Table 1.

Founder Line	Expression
511M	Induced expression in non-epithelial cells (with ROSA-Cre)
512M	Induced expression in non-epithelial cells (with ROSA-Cre)
513M	Expression in non-epithelial cells without induction
514F	N/A
515F	N/A
516M	N/A
517M	NO expression
518F	Expression in non-epithelial cells without induction
520F	NO expression

Table 1 XFP expression check in ROSA-Cre/+; PB-XFP animals

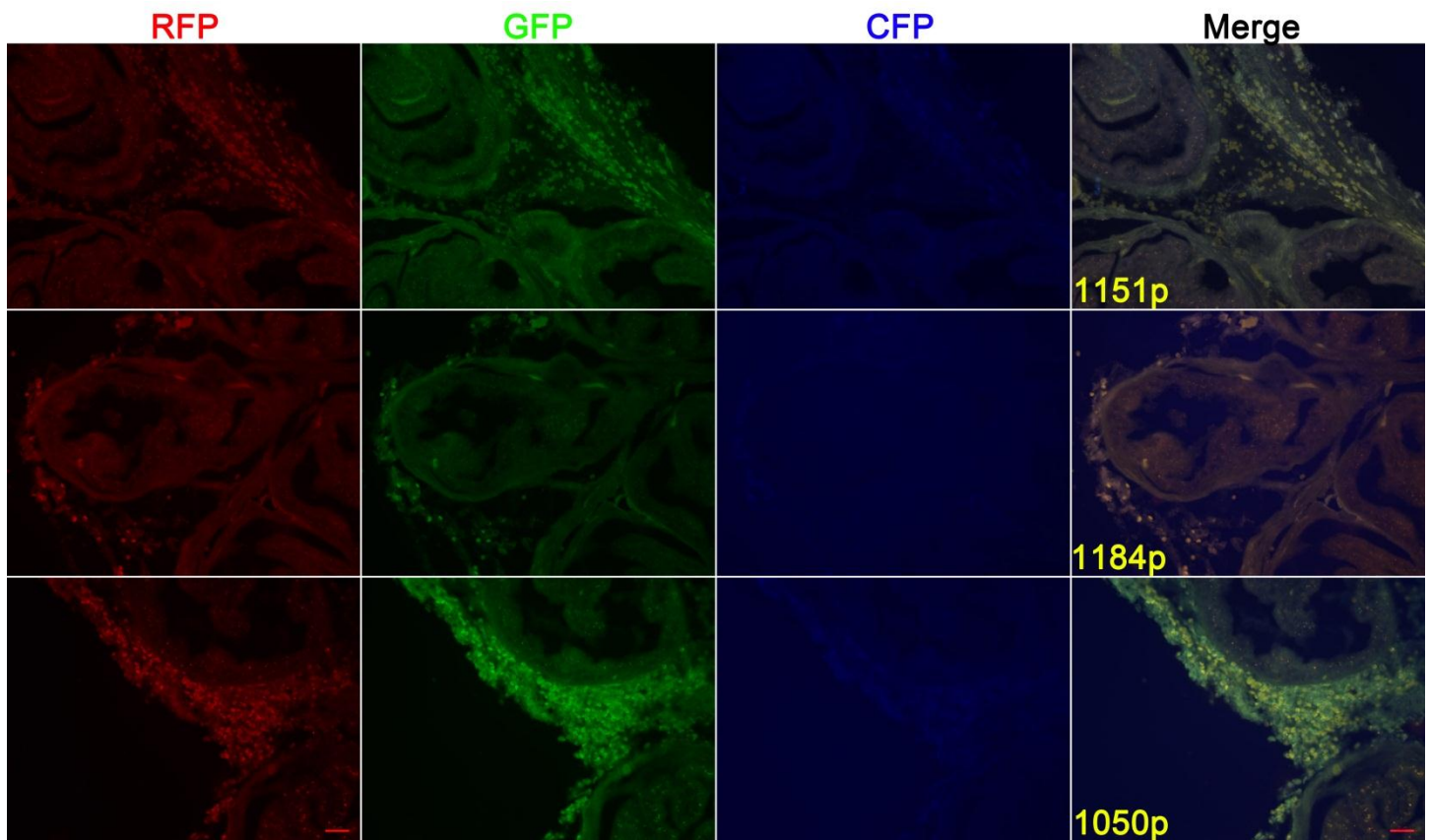


Figure 12 No epithelial XFP expression was observed in prostate tissue of ROSA-CreER; PB-XFP males induced with Tamoxifen. Paraffinized prostate sample were observed for endogenous fluorescent protein expression. #1151, #1184 and #1050 are male animals from line 511, 512 and 513, respectively. p stands for prostate tissue. Scale bar is 20 μ m.

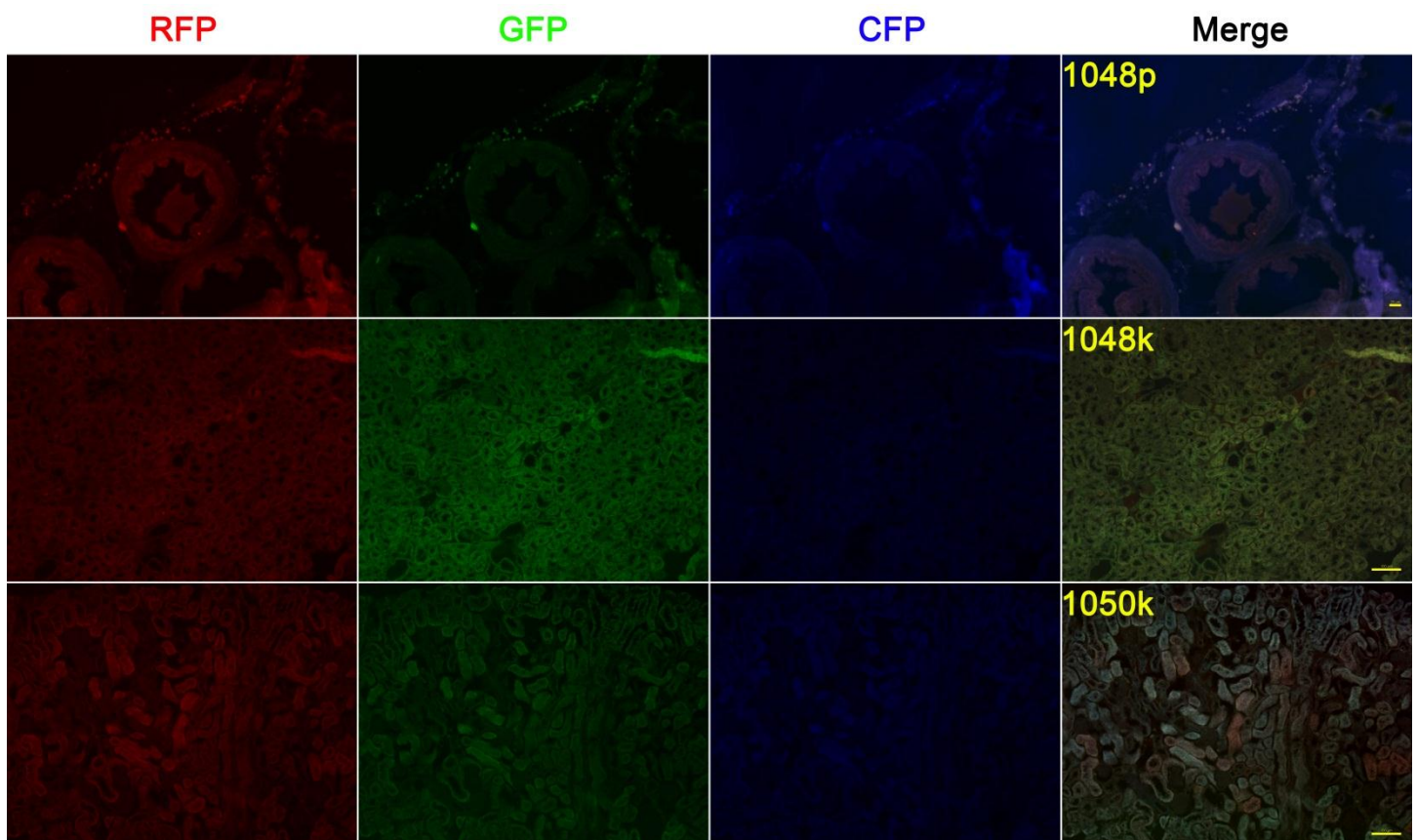


Figure 13 No epithelial XFP expression was observed in prostate or kidney tissue of ROSA-CreER; PB-XFP males that is not induced. Paraffinized prostate sample were observed for endogenous fluorescent protein expression. #1048 and #1050 are male animals from founder line 513. #1048 was non-induced, while #1050 was induced with Tamoxifen. p stands for prostate tissue. k stands for kidney tissue. Scale bar is 20µm.

For PB-Cre4 X CRK5-XFP breeding, there were fertility issues with founder CRK5-XFP animals with Pb-Cre males. Based on the difficulty with breeding, we switched to ROSA-Cre males, and we got three lines (#1015, 1016 and 1020) with ROSA-Cre; CRK5-XFP pups (Fig. 6E, 7B). Then those young pups were breeding with Pb-Cre males. We got Pb-Cre; CRK5-XFP male mice, and observed expression of CRK5-XFP in prostate gland from pups of line#1016 (Fig. 14). Interestingly, we observed fluorescent signals in both basal and luminal cells (Fig. 14B, C), as well as in stromal cells (Fig. 14C), indicating that basal cells could differentiate into luminal cells and stromal cells in normal prostate. The screening results were summarized in Table 2.

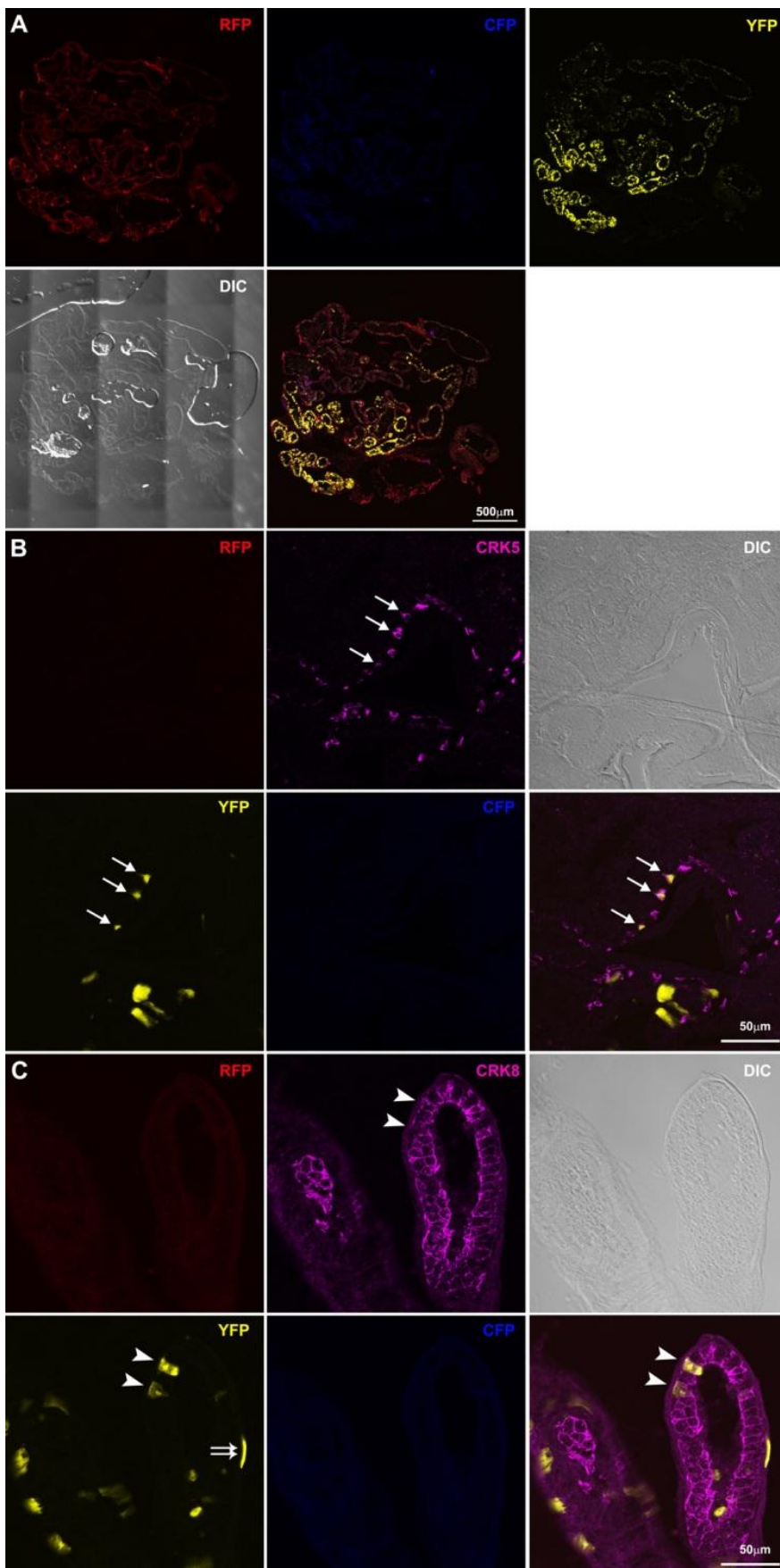


Figure 14 *Pb-Cre; CRK5-XFP* animals expressed XFP signals in prostate gland. A, XFPs were expressed in prostate cells, with YFP is the major expressing fluorescent protein. Scale bar, 500µm. B, XFPs were expressed in basal cells (arrows), which is marked by anti-CRK5 staining. Scale bar, 50µm. C, XFPs were expressed in luminal cells (arrowheads) and stromal cells (double-arrow). Luminal cells were marked by anti-CRK8 staining. Scale bar, 50µm.

Founder	Expression
1014F	N/A
1015F	No expression
1016M	Expression under PB-Cre control
1017M	N/A
1018M	N/A
1020M	No expression
1021M	N/A
1022M	N/A

Table 2 XFP expression check in PB-Cre4; CRK5-XFP animals

For PB-Cre4 x CMV-XFP breeding, we got several mice with PB-Cre; Brainbow 2.1^{lox/+} mice. We observed strong fluorescent signals in epithelial cells (Fig. 15, 16) and distinct subcellular localization of each kind of fluorescent protein (Fig. 16) by confocal fluorescent microscopy.

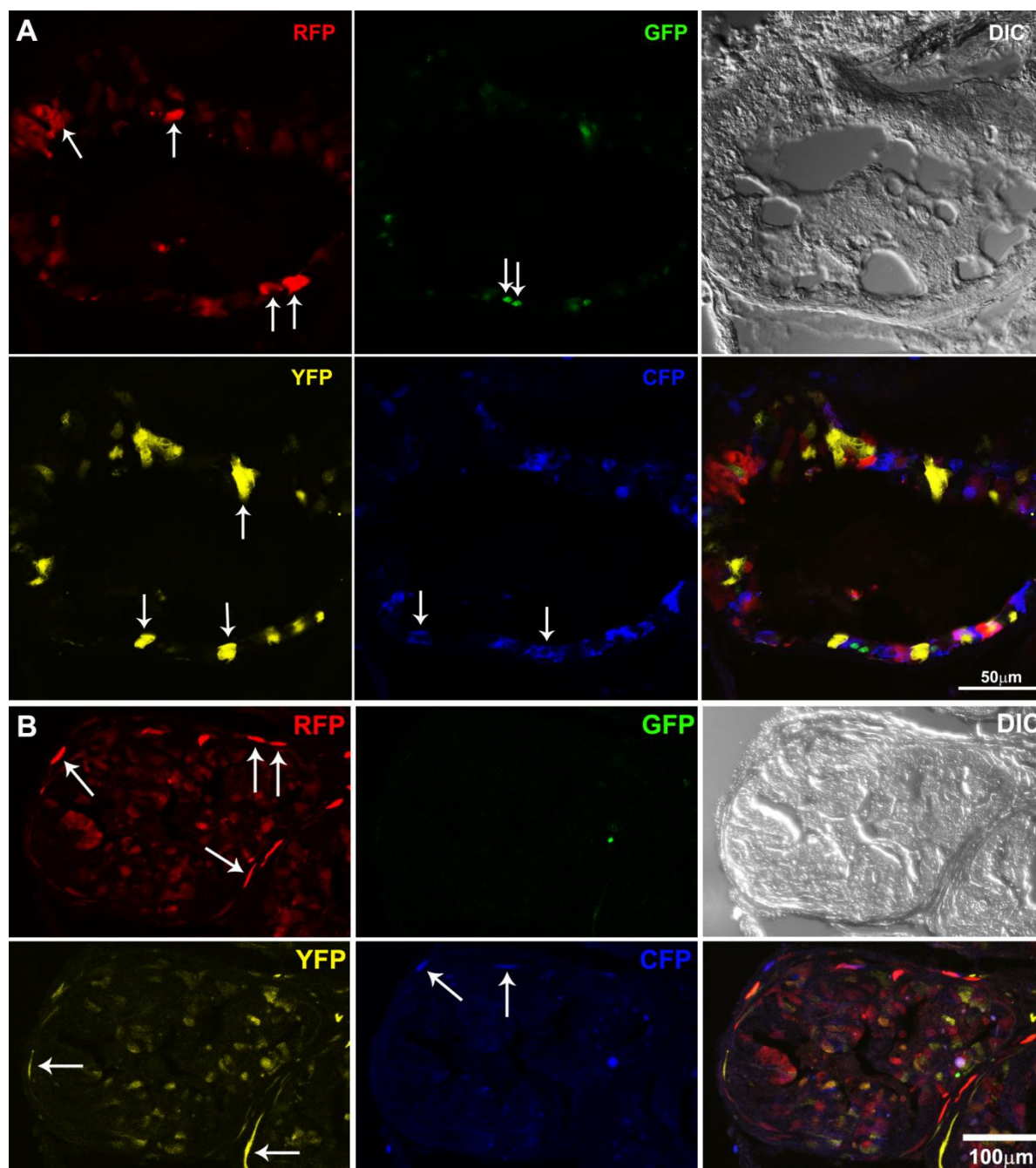


Figure 15 Fluorescent protein expression was observed in normal Prorainbow mouse prostate gland. A, frozen section of PB-Cre; Brainbow 2.1 lox/lox mouse prostate observed under confocal fluorescent microscope. As expected, RFP and YFP localize in cytoplasm, CFP on cell membrane, and GFP in nucleus (arrows). Scale bar, 50µm. B, Prorainbow signals were observed in prostate stromal cells (arrows). Scale bar, 100µm

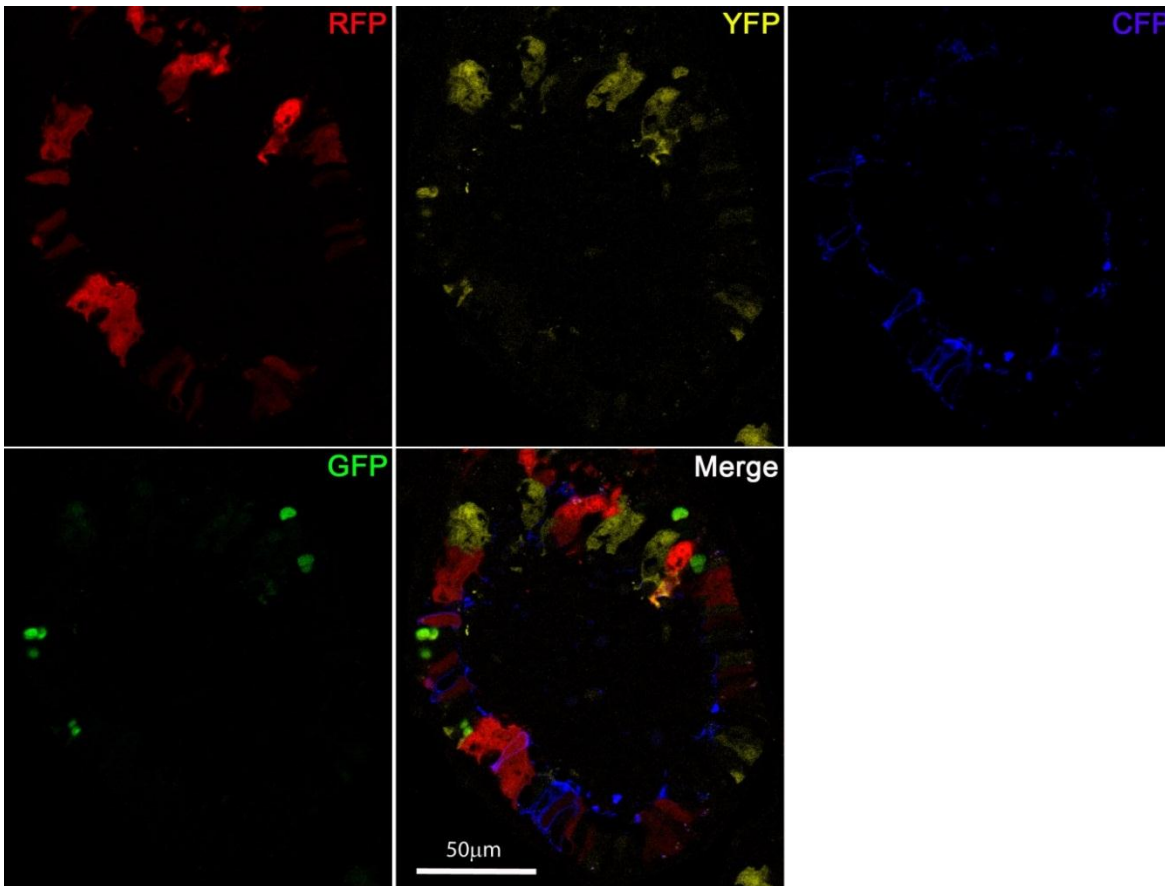


Figure 16 Fluorescent protein expressed in distinct cell compartment in normal Prorainbow mouse (PB-Cre; Brainbow 2.1 lox/+) prostate. As expected, RFP and YFP localize in cytoplasm, CFP on cell membrane, and GFP in nucleus. Scale bar, 50µm.

We also harvested other organs (lung, kidney and liver) and little XFP signal was detected in them (Fig 17). The results were confirmed with both IHC and endogenous protein check in frozen sections. We confirmed that the Prorainbow signal expression is strong and specific to prostate.

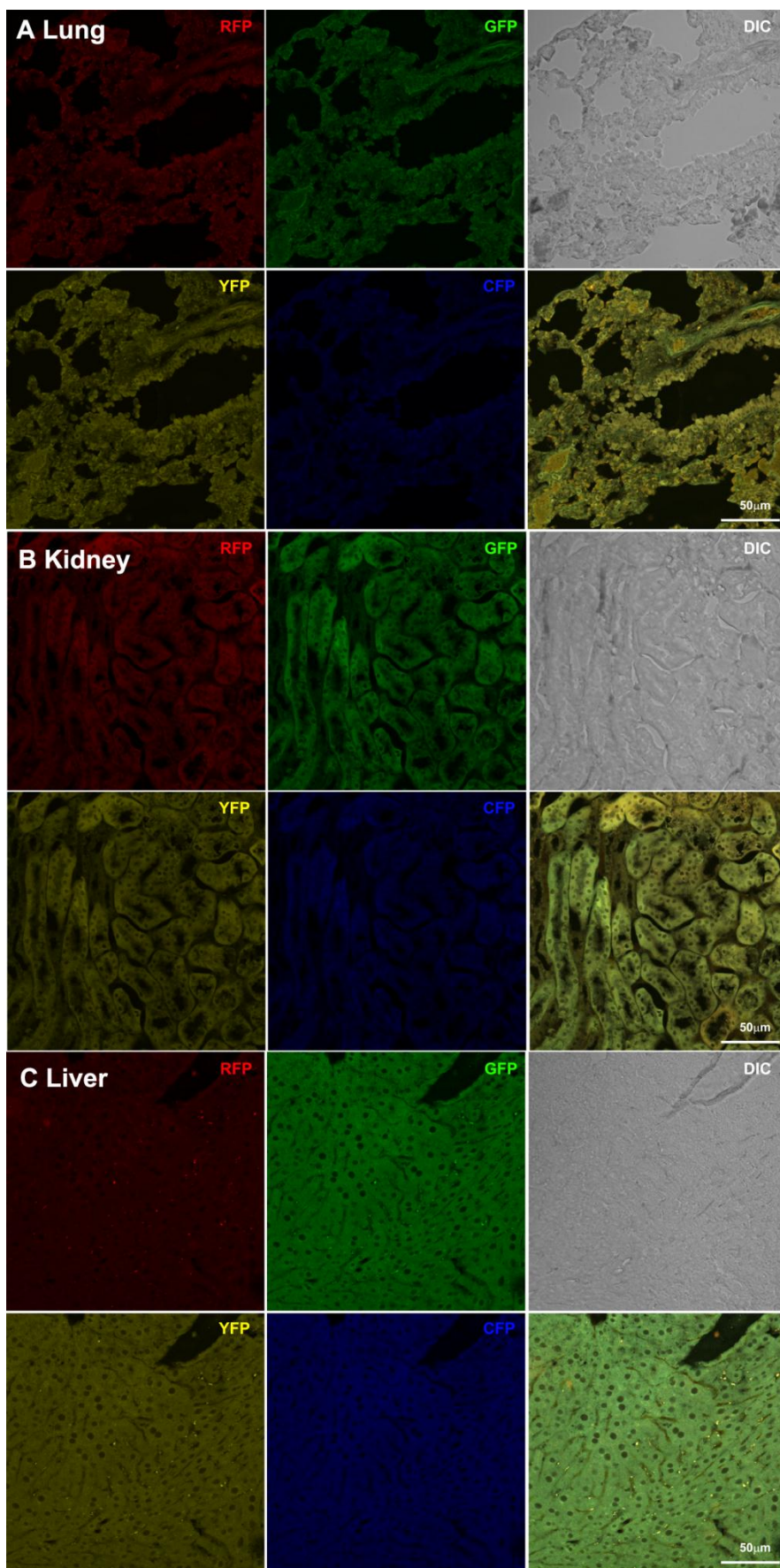
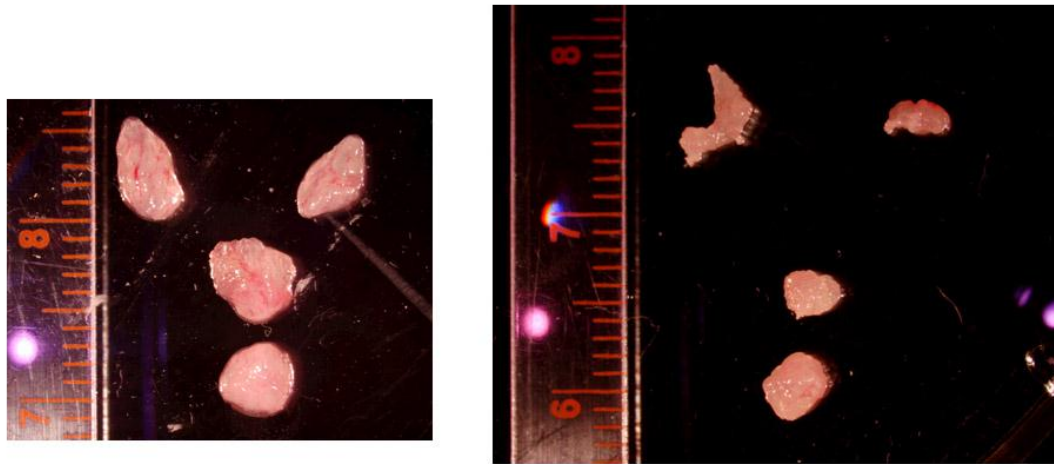


Figure 17 No XFP signal was observed in mouse organs other than prostate, such as lung (A), kidney (B) or liver (C).

Task II. Study of Prorainbow and PTEN or PKD1 knock-out mice hybrids.

We harvested prostate, kidney and lung from PB-Cre4; PKD1 lox/lox; CMV-XFP/+ animals (PKD1 knock-out with Brainbow 2.1 expression) and from PB-Cre4; PKD1 lox/lox; PTEN lox/lox; CMV-XFP/+ animals (PKD1 PTEN double knock-out with Brainbow 2.1 expression). In PKD1 knock-out animals, we didn't observe obvious size difference between prostate tissues from knock-out animal and those from control littermate (Fig. 18). The prostate tissue was labeled with different fluorescent colors in PKD1 knock-out prostate (Fig. 19).



PKD1 KO (195 days)

Control littermate (195 days)

Figure 18 Prostate tissues harvested from PKD1 knock-out animal and control litter mate. No obvious size difference was observed. Top, anterior lobes; middle, dorsal-lateral lobes; bottom, ventral lobe.

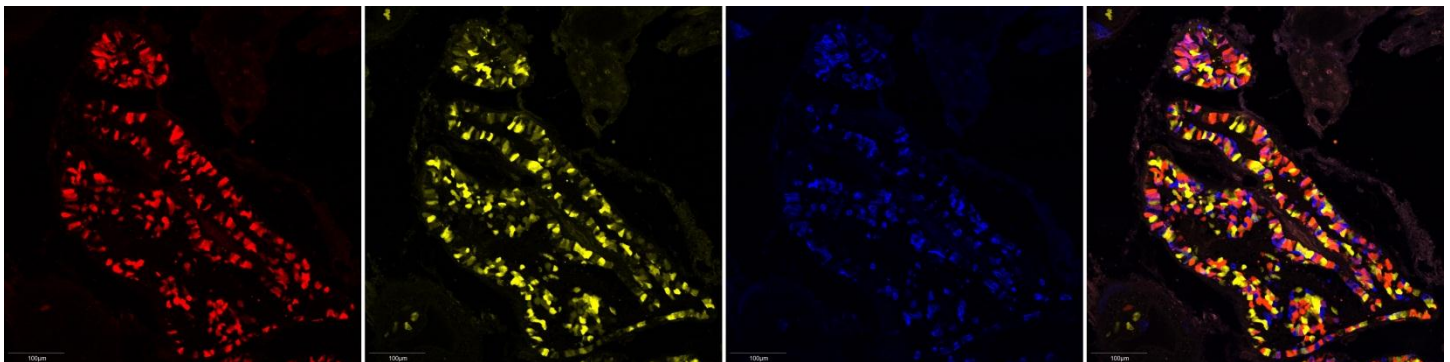


Figure 19 Fluorescent protein expression in frozen section of PB-Cre; PKD1^{lox/lox}; Brainbow 2.1^{lox/+} mouse prostate with confocal fluorescent microscopy. Scale bar, 100µm.

In PKD1 PTEN double knock-out animals, we observed obvious organ enlargement and local neoplasia in prostate, and the other organs (kidney, liver and lung) didn't show any sign of tumor formation (Fig. 20). We did histological check with H&E staining by comparing the double mutant prostate to control (Fig. 21). We also checked other organs (kidney, liver and lung) for invasive prostate cancer cells, but no invasion or metastatic lesion was observed at 8 months (222 days old) (Fig. 22). XFP expression in prostate epithelial cells was strong and specific in prostate of double knock-out animal. The expression pattern was patchy (Fig. 23), indicating a clonal origin of the cancer cells.

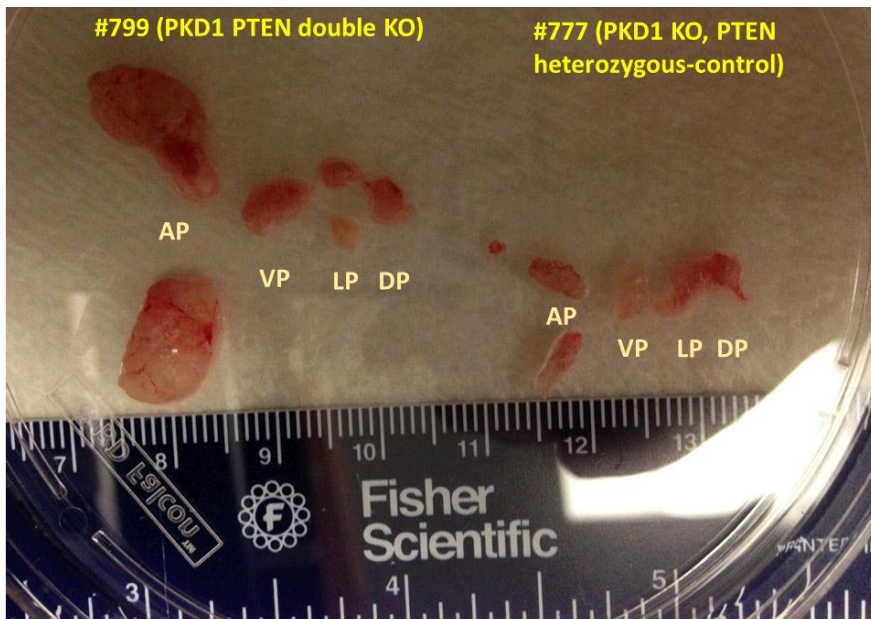
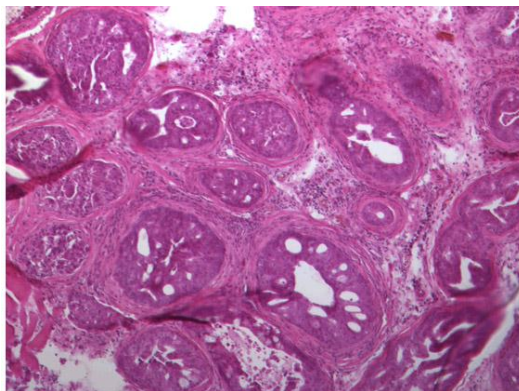
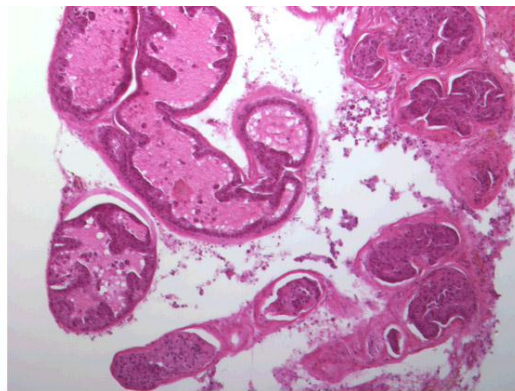


Figure 20 Prostate tissues harvested from PKD1 PTEN double knock-out animal and control litter mate. Prostate tissue from double knock-out animal was significantly increased. AP, anterior prostate; VP, ventral prostate; LP, lateral prostate; DP, dorsal prostate. Genotype of animal #799 is PB-Cre4; PKD1^{lox/lox}; PTEN^{lox/lox}; Brainbow 2.1^{lox/+}. Genotype of animal#777 is PB-Cre4; PKD1^{lox/lox}; PTEN^{lox/+}; Brainbow 2.1^{lox/+}. Animals were eight months old when prostate tissues were harvested.



#799 (PKD1 PTEN double KO)



#777 (control)

Figure 21 Histological analyses of prostate tissues from PKD1 PTEN double knock-out animal and control litter mate. Tissues were paraffinized and stained with H&E. Tumor progression and invasion were observed in double knock-out animals. Genotype of animal #799 is PB-Cre4; PKD1^{lox/lox}; PTEN^{lox/lox}; Brainbow 2.1^{lox/+}. Genotype of animal#777 is PB-Cre4; PKD1^{lox/lox}; PTEN^{lox/+}; Brainbow 2.1^{lox/+}. Animals were eight months old when prostate tissues were harvested.

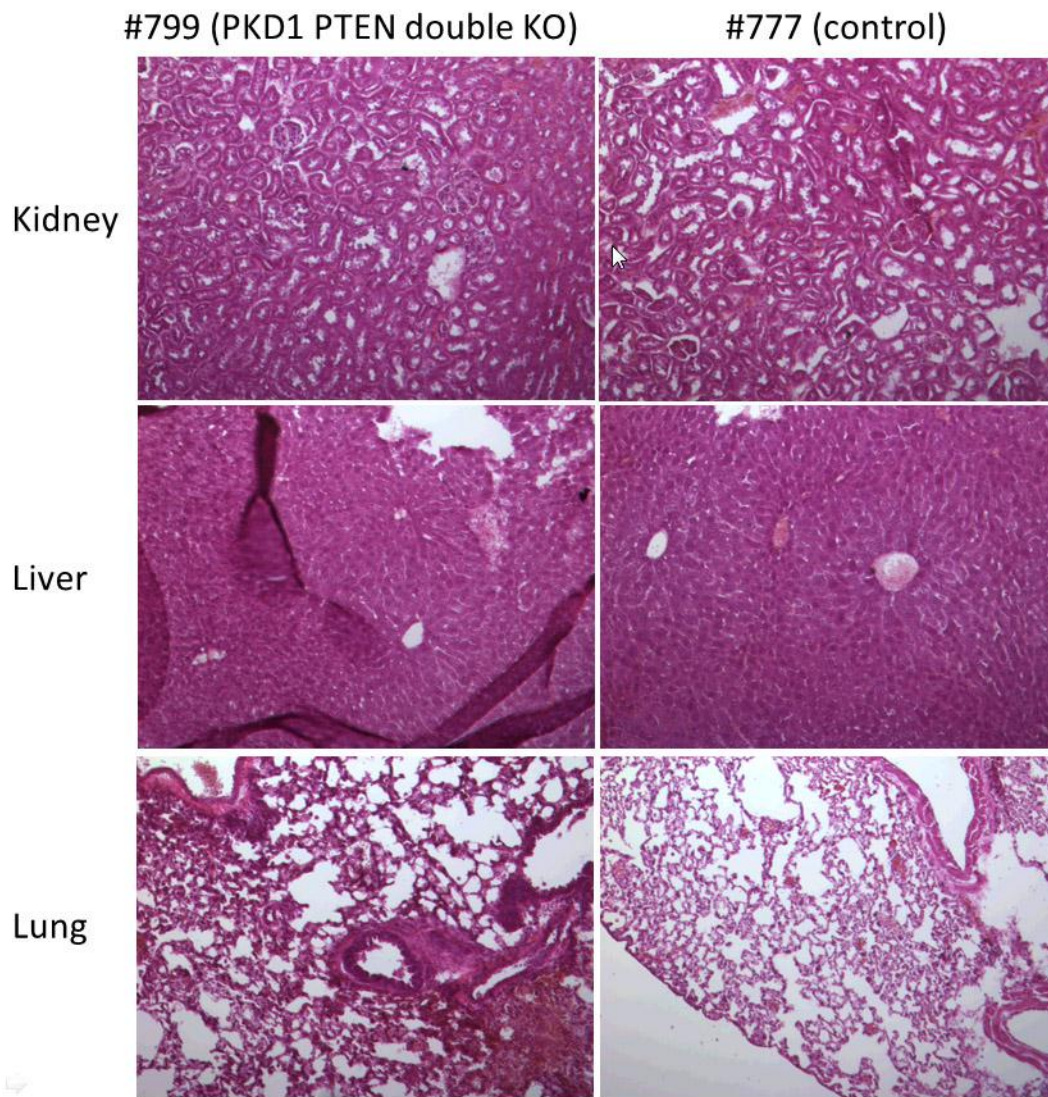


Figure 22 Histological analysis of lung tissues from PKD1 PTEN double knock-out animal and control litter mate. Tissues were paraffinized and stained with H&E. Tumor progression and invasion were observed in double knock-out animals. Genotype of animal #799 is PB-Cre4; PKD1^{lox/lox}, PTEN^{lox/lox}, Brainbow 2.1^{lox/+}. Genotype of animal #777 is PB-Cre4; PKD1^{lox/lox}, PTEN^{lox/+}, Brainbow 2.1^{lox/+}. Animals were eight months old when prostate tissues were harvested.

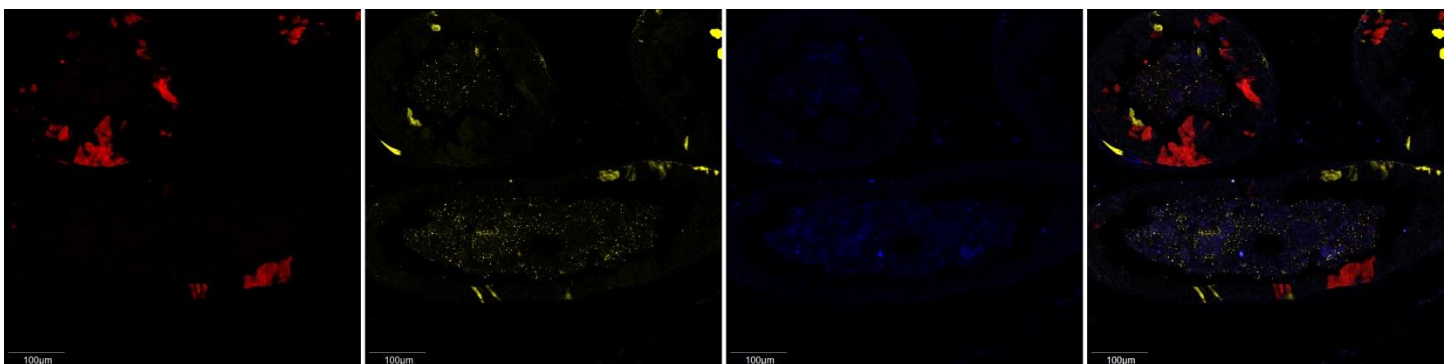


Figure 23 Fluorescent protein expression in frozen section of PB-Cre; PKD1^{lox/lox}; PTEN^{lox/lox}; Brainbow 2.1^{lox/+} mouse prostate with confocal fluorescent microscopy. Scale bar, 100µm.

Interestingly, quite a few cells lost XFP in prostate tissue in double knock-out animals (Fig. 23). In order to check whether the tumor cells could still be labeled with XFP signals, we did immunostaining with Ki67 protein (a cellular marker for proliferation), and by observation of Ki67 signal and endogenous XFP signals in tumor cells (Fig. 24), we confirmed that XFP expression was independent of cell proliferation.

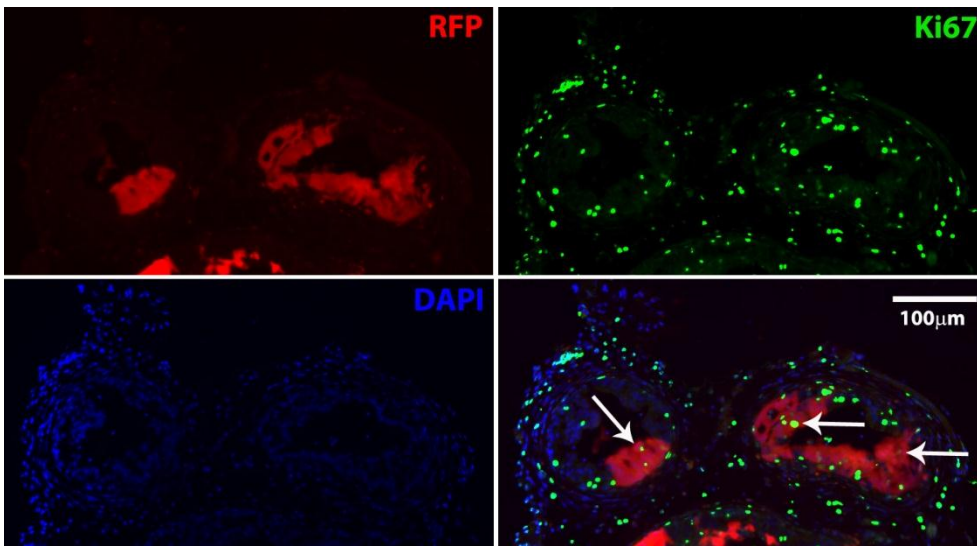


Figure 24 Fluorescent protein could label prostate tumor cells in double knock-out animal (genotype: PB-Cre; PKD1^{lox/lox}; PTEN^{lox/lox}; Brainbow 2.1^{lox/+}). Frozen section was stained with anti-Ki67 antibody and DAPI. Arrows indicate proliferating epithelial cells (cancer cells in prostate) that are labeled with fluorescent proteins. Scale bar, 100μm.

We also introduced Prorainbow labeling into K-Ras^{G12D} knock-in mouse, and observed that Prorainbow signals could label the hyperplasia tissues which resulted from K-Ras^{G12D} knock-in mutation (Figure 25). The XFP expression also displayed a patchy pattern, which indicated a clonal origin of hyperplastic cells.

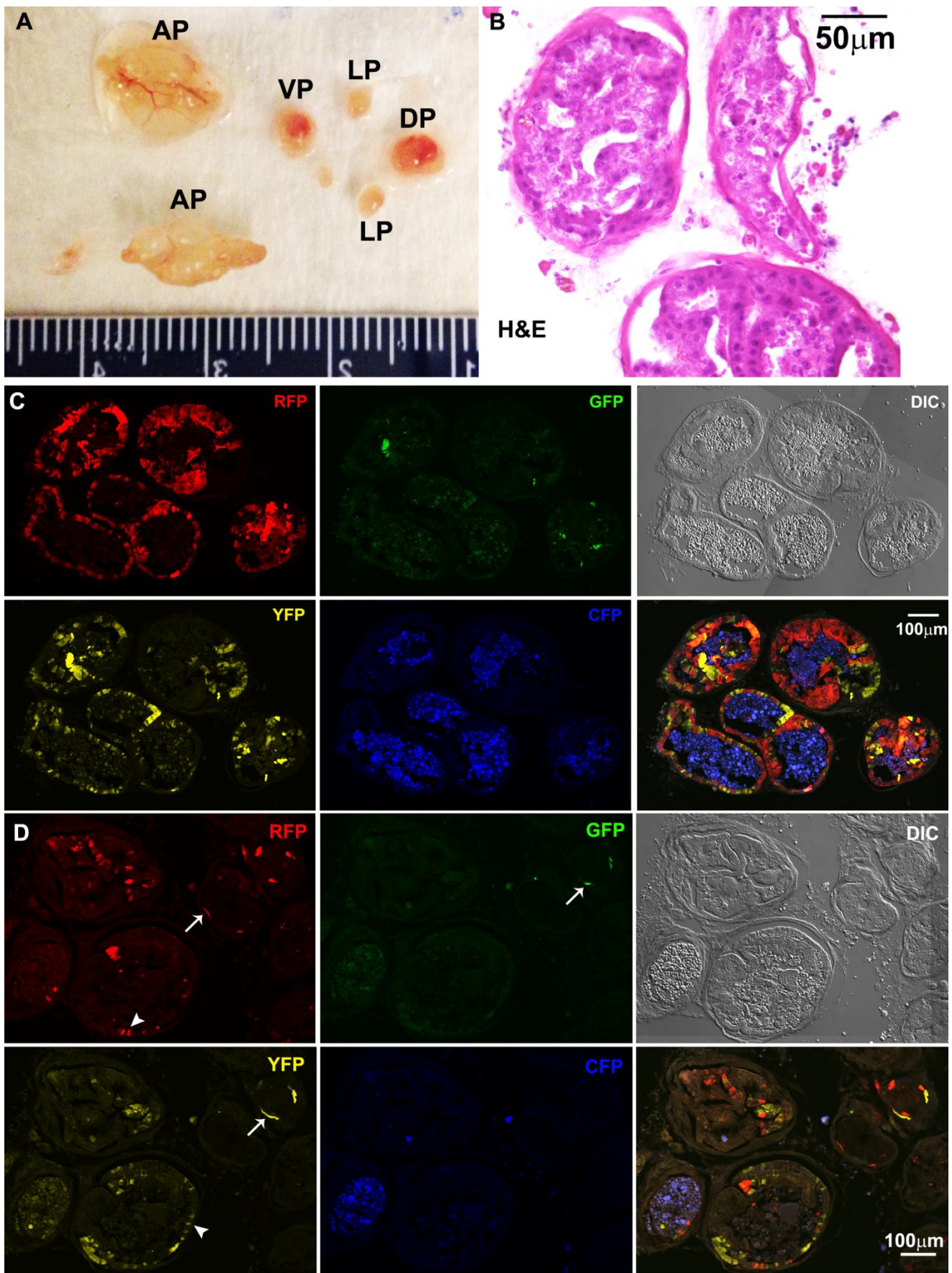


Figure 25 K-Ras^{G12D} over-expressing resulted in prostate hyperplasia. A, prostate tissues harvested from K-Ras^{G12D} Knock-in animal. AP, anterior prostate; VP, ventral prostate; LP, lateral prostate; DP, dorsal prostate. B, H&E staining of K-Ras^{G12D} over-expressing prostate gland. C and D, confocal images of endogenous XFPs in K-Ras^{G12D} over-expressing prostate gland. XFPs expressed in both epithelial cells (arrowheads) and stromal cells (arrows). Scale bar, 100μm.

Finally, we generated K-Ras^{G12D} knock-in (KI) PTEN knock-out (KO) double mutant animals, and checked the expression of Prorainbow signals in prostate. We found that prostate gland was enlarged in double mutant animals (Fig. 26). We found that K-Ras^{G12D} KI PTEN KO double mutant animal develop malignant carcinoma in prostate gland (Fig. 27A, B), and local invasion into urethra was observed (Fig. 27C, D). Both the tumor development and invasion was labeled by Prorainbow signals (Fig. 27).

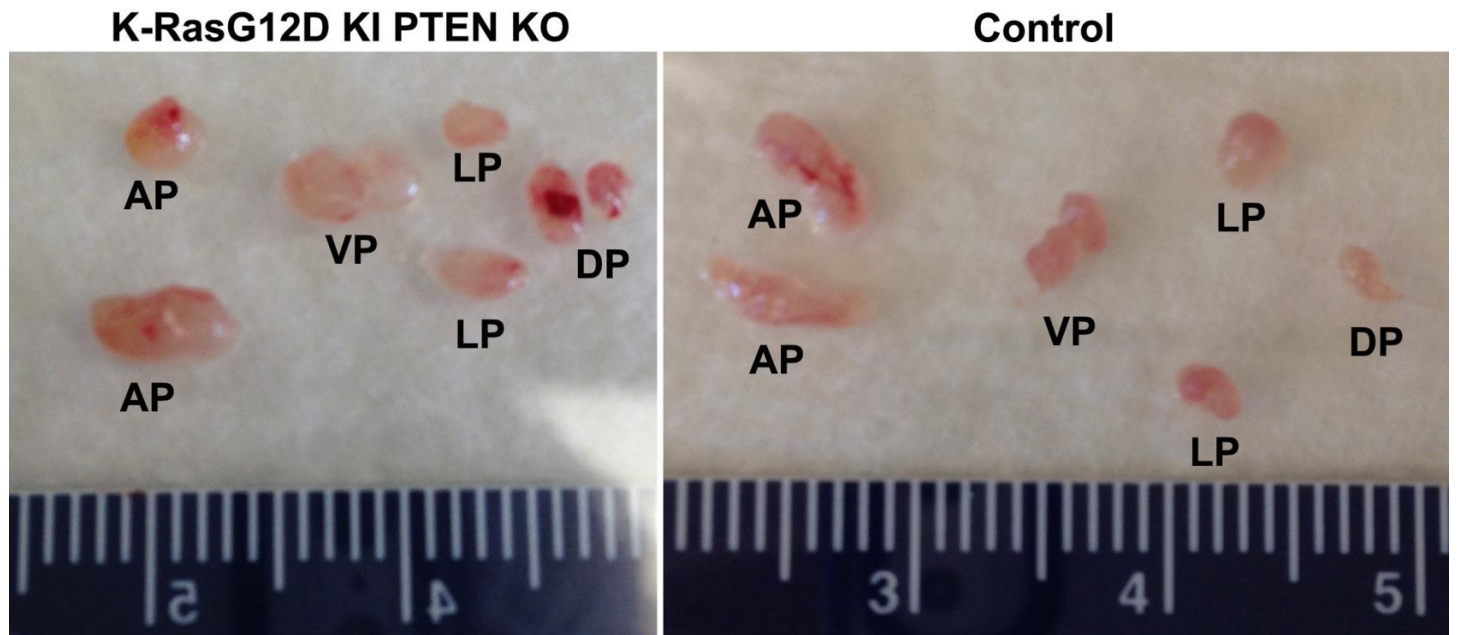


Figure 26 Prostate gland was harvested from double mutant animal (60 days old) and control litter mate (75 days old).

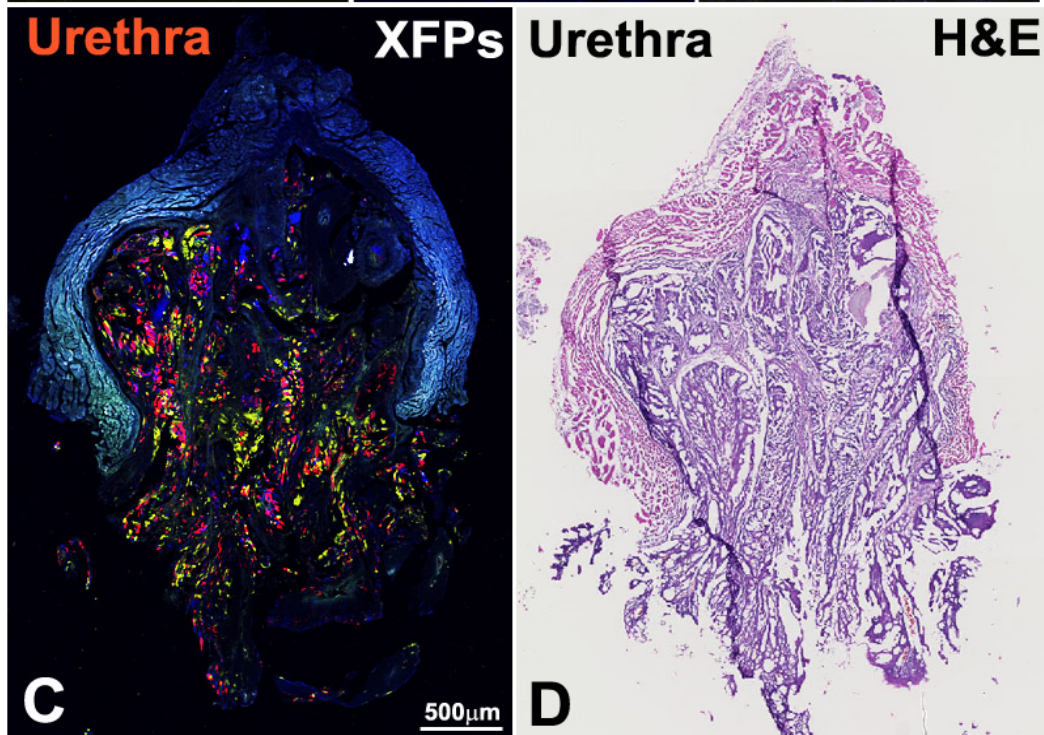
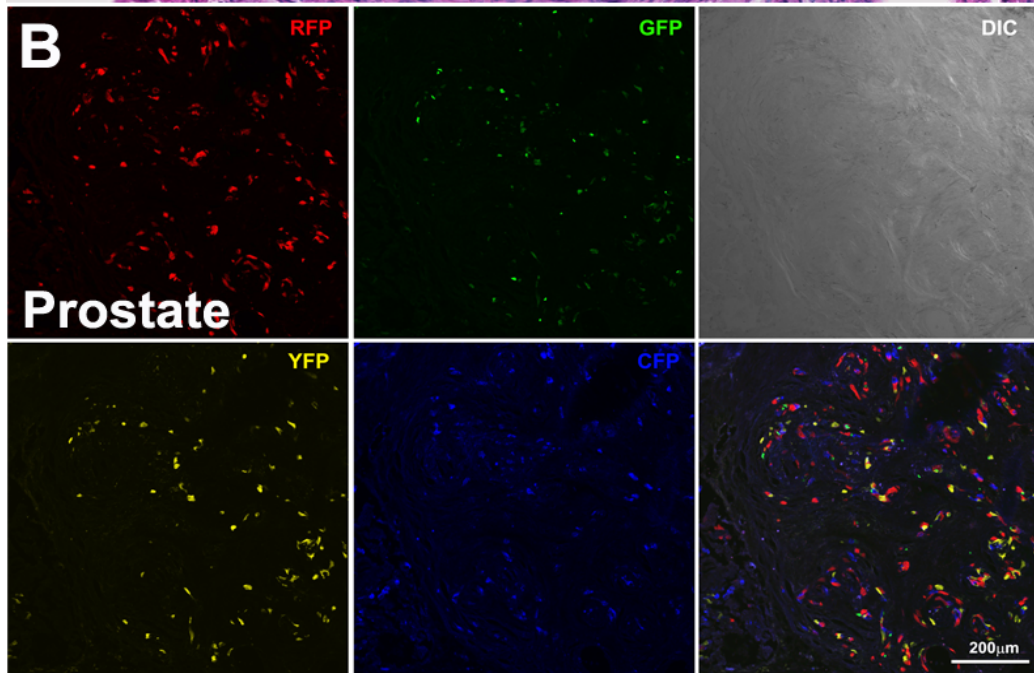
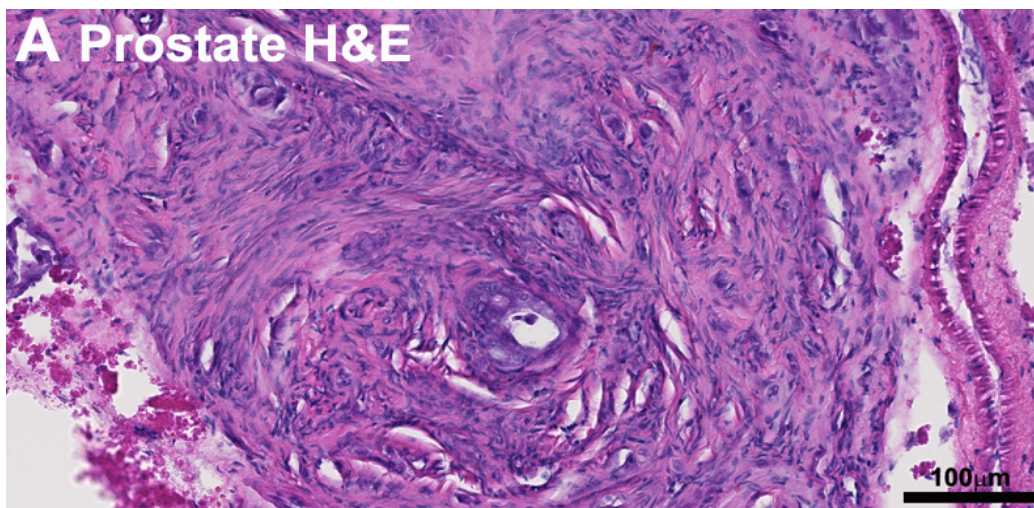


Figure 27 Fluorescent proteins could label prostate tumor cells in K-Ras^{G12D} Knock-in PTEN knock-out double mutant animal (genotype: PB-Cre; PKD1^{lox/+}; K-Ras^{G12D lox/+}; PTEN^{lox/lox}; Brainbow 2.1^{lox/+}). A, histological analysis of prostate gland from K-Ras PTEN double mutant animal by H&E staining. B, Prorainbow signals observed in prostate from K-Ras PTEN double mutant animal by confocal microscopy. C, histological analysis of urethra from K-Ras PTEN double mutant animal by H&E staining. D, Prorainbow signals observed in urethra from K-Ras PTEN double mutant animal by confocal microscopy, indicating the local invasion of prostate cancer cells.

As a summary, we successfully produced prostate specific PKD1 knock-out, K-Ras^{G12D} knock-in, PKD1 PTEN double knock-out and K-Ras^{G12D} knock-in PTEN knock-out double mutant mice with CMV-XFP (Brainbow 2.1) fluorescent signal in prostate. We confirmed that XFP signals could express specifically in prostate under PB-Cre4 control, and could label normal, hyperplastic, neoplastic and malignant carcinoma cells. More importantly, the invasive cells in other tissue (e.g. urethra) could be labeled clearly by Prorainbow signals, suggesting the potential application in more advanced prostate cancer models for metastasis detection. We didn't observe any sign of tumor progression in PKD1 knock-out animals at eight months, but we did see an obvious enlargement of prostate tissue in PKD1 PTEN double knock-out animal. The patchy expression pattern of XFPs in K-Ras^{G12D} knock-in or PKD1 PTEN double knock-out prostates suggested a clonal origin of hyperplastic and local neoplastic cells. We confirmed that the loss of XFP expression in PKD1 PTEN double knockout prostate is independent of cell proliferation. In K-Ras^{G12D} knock-in PTEN knock-out double mutant animals, we observed malignant carcinoma development in prostate gland, as well as local invasion into urethra, which was confirmed by both endogenous fluorescent labeling and H&E staining. Invasive prostate cancer cells were labeled clearly by XFP signals. For transgenic lines developed in our lab, we tested PB-XFP and CRK5-XFP transgene expression in transgenic lines from 17 founder animals, and generated one CRK5-XFP transgenic line, which successfully expresses Prorainbow signals in prostate. Expression of CRK5-XFP was observed in basal cells of normal prostate gland. Interestingly, we also observed XFPs in luminal cells and stromal cells, indicating that they could be differentiated from basal cells. Due to the time limitation we didn't induce CRK5-XFP expression in PKD1 PTEN double knock-out mouse or K-Ras^{G12D} knock-in PTEN knock-out double mutant mouse. We hope we could use this transgenic line to discover the cell origin of malignant cancer and of metastasis in future.

For training and professional development, results disseminated to communities of interest, and plan for the next reporting period, there is nothing to report.

Reference

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA: A Cancer Journal for Clinicians. 2013;63(1):11-30.
2. Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, et al. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature. 2007;450(7166):56-62.

Impact

This study has created and established the utility of Prorainbow mouse model to study the prostate. Because stochastic fluorescent labelling is seen fairly uniformly in benign prostate and patchier in hyperplastic and neoplastic prostate, the model could be used to discriminate different pathologies in prostate in addition to studying clonal origins of cells. Concurrent development of newer technologies will increase the application of Prorainbow mouse model in animal research. The model has a potential to become a cheap and viable tool to address specific questions in cancer biology such as cellular origin of cancer and study of metastatic cascade among others.

Changes/Problems

Due to the low fertility rate of CRK5-XFP animals, we increased one more step in breeding by crossing them with ROSA-Cre animals, so as to keep the transgenic line.

Products

1. 'Novel *In vivo* Model for Combinatorial Fluorescence Labeling in Mouse Prostate', Xiaolan Fang, Kenneth Gyabaah, Bitu Nick Kholgh, J. Mark Cline, K.C. Balaji, *Prostate*, 2014, Submitted.
2. 'Novel *in vivo* Prostate Mouse Models to Study Origin of Prostate Cancer', Xiaolan Fang, Prostate Cancer Research and Translation Symposium, 2014, invited oral presentation.

3. 'Tracking the Origin of Prostate Cancer', Xiaolan Fang, Wake Forest Postdoctoral Association (PDA) Seminar Series, 2013, invited seminar.

Participants and other collaborating organizations

Name	BALAJI, KETHANDAPATTI C
Project role	Principle Investigator
Nearest person month worked	12
Contribution to project	Dr. Balaji has led the research projects and oversaw the progress.

Name	FANG, XIAOLAN
Project role	Research Fellow
Nearest person month worked	12
Contribution to project	Dr. Fang has performed the work of experimental design, genotyping, immunohistochemistry, mouse breeding, mouse management, mouse surgery, mouse tissue preservation and processing, confocal fluorescent microscopy and data analysis.
Funding Support	National Institute of Health (Grant#CA079448)

Name	GYABAAH, KENNYTH
Project role	Technician
Nearest person month worked	3
Contribution to project	Mr. Gyabaah has performed the work of genotyping, molecular cloning, mouse tissue preservation and processing, immunohistochemistry and confocal fluorescent microscopy.

Name	SINK, SANDY
Project role	Technician
Nearest person month worked	3
Contribution to project	Ms. Sink has performed the work of mouse breeding and tail snipping.

Name	NICKKHOLGH, BITA
Project role	Postdoc Fellow
Nearest person month worked	3
Contribution to project	Dr. NickKholgh has performed the work of genotyping, mouse surgery and mouse tail snipping.

Name	WICKS, ELIZABETH
Project role	Summer Intern (Undergraduate Student)
Nearest person month worked	2.5
Contribution to project	Ms. Wicks has performed the work of genotyping, immunohistochemistry and confocal microscopy.
Funding Support	Wake Forest Institute for Regenerative Medicine (WFIRM) Summer Scholars Program

Special reporting requirements

N/A

Appendix1



REPLY TO
ATTENTION OF

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

July 05, 2011

Director, Office of Research Protections
Animal Care and Use Review Office

Subject: Review of USAMRMC Proposal Number PC094003, Award Number W81XWH-10-1-0709 entitled, "Tracking Origins of Prostate Cancer: An Innovative In Vivo Modeling"

Principal Investigator Kethandapatti Balaji
Wake Forest University Health Sciences (WFUHS)
Winston-Salem, NC

Dear Dr. Balaji:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Programs"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, protocol PC094003 entitled, "Tracking Origins of Prostate Cancer-An Innovative In Vivo Model," IACUC protocol number A11-097 is approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of mice and will remain so until its modification, expiration or cancellation. This protocol was approved by the Wake Forest University IACUC.

When updates or changes occur, documentation of the following actions or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)
- USDA annual program/facility inspection reports
- Reports to OLAW involving this protocol regarding
 - a. any serious or continuing noncompliance with the PHS Policy;
 - b. any serious deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or
 - c. any suspension of this activity by the IACUC
- USDA or OLAW regulatory noncompliance evaluations of the animal facility or program
- AAALAC, International status change (gain or loss of accreditation only)

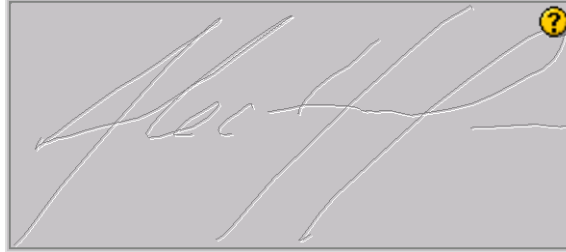
Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

- Species used (must be approved by this office)
- Number of each species used

- USDA Pain Category for all animals used

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: acuro@amedd.army.mil.

Sincerely,

A rectangular box containing a handwritten signature in black ink. The signature appears to be "Alec Hail". In the top right corner of the box, there is a small yellow circular icon with a black question mark inside.

Alec Hail, DVM, DACLAM
Colonel, US Army
Director, Animal Care and Use
Review Office

Copies Furnished:

Ms. Amber Stillrich, US Army Medical Research Acquisition Activity (USAMRAA)
Dr. Nrusingha Mishra/MCMR-PLF
Dr. David Lyons, Wake Forest University
Ms. Amy L. Comer, Wake Forest University